

Distribution of Nitrogen During the Embryonic Development of the Mealworm, *Tenebrio molitor* Linnaeus¹

ROBERT P. KELLY² AND DANIEL LUDWIG

DEPARTMENT OF BIOLOGICAL SCIENCES, FORDHAM UNIVERSITY

Abstract: During embryogenesis of the mealworm, total nitrogen remains constant at 1.27 mg./100 eggs. Approximately 24% of the total was converted from water soluble to water insoluble material. The utilization of albumin accounted for almost 50% of this material. An increase in globulin accounted for 25% of the change in water insoluble protein, while synthesis of scleroprotein accounted for 30%. The remaining materials were not defined by the procedures employed.

The period of embryogenesis involves extensive changes in nitrogenous compounds. Protein metabolism is important because it is involved in the formation of structural elements and enzyme systems. Nitrogenous compounds may also be used for energy metabolism (Needham 1931, 1942).

Farkas (1903) working on the silkworm, *Bombyx mori*; Horowitz (1939), on the geophyean worm, *Urechis caupo*; Trowbridge and Bodine (1940), on the grasshopper, *Melanoplus differentialis*; and Rothstein (1952), on the Japanese beetle, *Popillia japonica*, found no measureable change in total nitrogen content during embryogenesis. This consistency indicates that proteins of the insect embryo are constructed from nitrogenous substances present in the egg at the beginning of development. During the embryonic period, proteins may be formed by synthesis from low molecular weight precursors or by the transformation of egg protein. This transformation may be direct or involve the catabolism of egg proteins.

Several investigators have fractionated the nitrogenous compounds at various stages of development to study transformations occurring during the developmental period. These studies have yielded a small number of chemically ill-defined fractions. Pigorini (1925) worked on changes in the distribution of nitrogen at several stages of embryogenesis in the silkworm, *B. mori*. His results showed that the albumin and mucoprotein fractions decreased sharply during the last seven days of embryogenesis. These changes were complementary to an increase in the globulin fraction occurring during the same period. Horowitz (1939) described the changes in protein, peptide, non-protein, and amino nitrogen during the development of the egg of *U. caupo*. He reported a 6.4% increase in protein nitrogen due to shifts of material from the amino and peptide fractions.

¹ Dissertation submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology at Fordham University.

² Present address: Department of Biology, St. Peter's College, Jersey City, N. J.

He explained this very slight increase by assuming that there is a continuous breakdown of yolk proteins followed by resynthesis into the proteins of the embryo. Ludwig and Rothstein (1952) noted that these changes might have been greater if trichloroacetic acid (TCA) had not been used as a killing agent prior to extraction. They studied the distribution of nitrogen during the embryonic development of the Japanese beetle, *P. japonica*. Approximately 80% of the total nitrogen of the newly laid egg was contained in the water soluble compounds precipitated by tungstic acid. Most of this material was incorporated into insoluble protein as development progressed. In their procedure, the egg was extracted with an alcohol-ether solution followed by boiling water previous to the separation of the tungstic acid precipitate. DeVecchio (1955) demonstrated that this treatment can cause a shift of material into the insoluble fraction. This apparently did not occur in the Japanese beetle egg.

Differences in various procedures do not allow for a clear comparison of results. For this reason the following study of the nitrogenous composition of the mealworm, *Tenebrio molitor*, was initiated. It includes fractionations of the egg on each day of development by three different procedures and allows for a more meaningful interpretation of earlier work.

MATERIALS AND METHODS

Newly emerged adults were collected from stock cultures maintained at 25°C. on chick growing mash. The beetles were placed in bowls containing white flour and maintained at 25°C. A bottle of water plugged with moist cotton was placed in each culture. Eggs were collected by sifting the flour at 24-hour intervals from cultures of approximately 1 to 4 weeks of age. They were transferred to a humidifier containing a saturated solution of NaCl (relative humidity 76%) and incubated at 25°C. At the desired stage of development, 100 eggs were removed, placed in a 15 ml. calibrated centrifuge tube, crushed with a glass rod, and immediately vacuum desiccated. They were stored under vacuum desiccation and tested within the following 24-hour period. All measurements were made on samples of 100 individuals at the following stages of development; newly laid, one, two, three, four, five, six, seven day eggs, and newly emerged larvae plus chorions (day 8).

Three fractionation procedures were employed. In all cases, the samples were removed from the vacuum desiccator and powdered with a glass rod previous to subsequent fractionation. The nitrogen content of each fraction was determined by the semimacro-Kjeldahl procedure described by Niederl and Niederl (1938) as modified by Wagner (1940). All fractionation procedures were performed at room temperature.

Using the method of Ludwig and Rothstein (1952), the nitrogenous compounds were divided into four fractions: lipid nitrogen (Fraction A); water

soluble nitrogen not precipitated by TCA (Fraction B); water soluble nitrogen precipitated by TCA (Fraction C); and insoluble nitrogen (Fraction D).

The material was also fractionated by the method of DelVecchio (1955). This procedure is similar to the above, however, the order of fractionation is changed with the removal of water soluble materials preceding the lipid extraction. Furthermore, water at 25°C. was employed in place of boiling water for the aqueous extraction. Four fractions corresponding to the fractions of Ludwig and Rothstein were obtained by this procedure.

A seven fraction technique was developed as an extension of the method of DelVecchio. The water soluble material precipitated by TCA (DelVecchio Fraction C) was separated into two fractions on the basis of heat coagulation. The water insoluble material (DelVecchio Fractions A and D) was divided into four fractions. In this procedure, 8 ml. of distilled water were added to a 15 ml. centrifuge tube containing the powdered sample and the material was suspended by stirring with a glass rod. The suspension was allowed to stand, with frequent stirring, for ten minutes, centrifuged, and the supernate decanted into another 15 ml. centrifuge tube. This extraction was repeated and the supernate decanted into a third 15 ml. centrifuge tube. The two tubes containing the water extract were placed in a boiling water-bath for 30 minutes, centrifuged, and the supernates transferred into two 15 ml. centrifuge tubes. The precipitates were transferred quantitatively to a digestion flask and the nitrogen content determined as Fraction A (water soluble nitrogen precipitated by boiling). Five ml. of 30% TCA were added to each tube containing the supernates obtained after heat coagulation of the water extracts. The contents of the tubes were stirred and allowed to stand for 30 minutes, centrifuged, and the supernates decanted into a 100 ml. digestion flask. The residues were washed with several ml. of 30% TCA, centrifuged, and the supernates added to that already present in the digestion flask. The nitrogen content was then determined as Fraction B (water soluble nitrogen not precipitated by boiling or by TCA). The TCA precipitates were transferred quantitatively to a digestion flask and the nitrogen content determined as Fraction C (water soluble nitrogen precipitated by TCA).

Ten ml. of a 10% NaCl solution were added to the residue remaining after the water extraction. The suspension was allowed to stand, with frequent stirring, for 10 minutes, centrifuged, and the supernate decanted into a 100 ml. digestion flask. This extraction was repeated and the supernate added to that already present in the flask. The nitrogen content of this fraction was determined as Fraction D (water insoluble nitrogen extracted with 10% NaCl). Ten ml. of a 0.1N NaOH solution were then added to the residue remaining after the salt extraction. The suspension was allowed to stand, with frequent stirring, for 10 minutes, centrifuged, and the supernate decanted into a 100 ml. digestion flask. The residue was resuspended in 10 ml. of 0.1N HCl and allowed to stand

TABLE 1. Distribution of nitrogen during the embryogenesis of mealworm (technique of Ludwig and Rothstein). Figures, expressed as per cent total nitrogen, are given with standard errors.

| Age | No. of trials | Fraction A | Fraction B | Fraction C | Fraction D |
|---|---------------|-----------------|-----------------|-----------------|------------------|
| Newly laid eggs | 10 | 8.6 ± 0.146 | 7.0 ± 0.122 | 7.4 ± 0.203 | 77.0 ± 0.142 |
| 1 day | 8 | 8.4 ± 0.141 | 6.7 ± 0.208 | 7.0 ± 0.106 | 77.9 ± 0.356 |
| 2 day | 8 | 7.8 ± 0.303 | 6.7 ± 0.208 | 6.8 ± 0.124 | 78.7 ± 0.153 |
| 3 day | 8 | 7.0 ± 0.191 | 6.4 ± 0.207 | 6.6 ± 0.166 | 80.0 ± 0.241 |
| 4 day | 8 | 7.0 ± 0.148 | 6.8 ± 0.106 | 5.7 ± 0.140 | 80.5 ± 0.291 |
| 5 day | 8 | 7.1 ± 0.153 | 6.5 ± 0.202 | 5.7 ± 0.166 | 80.7 ± 0.280 |
| 6 day | 8 | 6.7 ± 0.131 | 6.7 ± 0.138 | 5.8 ± 0.146 | 80.8 ± 0.134 |
| 7 day | 8 | 6.7 ± 0.161 | 6.3 ± 0.115 | 6.0 ± 0.113 | 81.0 ± 0.197 |
| Newly hatched larvae + chorions (Day 8) | 8 | 6.8 ± 0.181 | 5.7 ± 0.122 | 5.7 ± 0.130 | 81.8 ± 0.238 |

for 10 minutes, centrifuged, and the supernate added to that already present in the digestion flask. The nitrogen content of this fraction was determined as Fraction E (water insoluble nitrogen extracted with 0.1N NaOH or 0.1N HCl). A solution consisting of 1 ml. distilled water, 4.5 ml. absolute ethanol, and 4.5 ml. absolute ethyl ether was mixed with the residue remaining after the base-acid extraction and allowed to stand, with frequent stirring, for 30 minutes, centrifuged, and the supernate decanted into a 100 ml. digestion flask. The residue was suspended in another 10 ml. of alcohol-ether solution, centrifuged, and the supernate added to that already present in the digestion flask. The ether and most of the alcohol were evaporated, 25 ml. of distilled water added, and the nitrogen content determined as Fraction F (water insoluble nitrogen extracted with lipid solvents). The residue remaining after removal of the alcohol-ether extraction was transferred quantitatively to a digestion flask and its nitrogen content determined as Fraction G (insoluble nitrogen).

OBSERVATIONS

The nitrogen content per 100 eggs remained constant at 1.27 mg. during the seven days of development. There was a decrease to 1.10 mg. at hatching, associated with the loss of chorion.

The nitrogen content for the fractions obtained by the technique of Ludwig and Rothstein are given in Table 1. Each fraction is expressed as per cent total nitrogen. Fraction A (lipid nitrogen) decreased from 8.6 to 6.8% through the embryonic period with significant (95% level of confidence) decreases on the third and sixth day. Fraction B (water soluble nitrogen precipitated by TCA) remained relatively constant at approximately 6.8% during the first 6 days with significant decreases to 6.3 during the seventh and to 5.7% on the eighth

TABLE 2. Distribution of nitrogen during the embryogenesis of the mealworm (technique of DelVecchio). Figures, expressed as per cent total nitrogen, are given with standard errors.

| Age | No. of trials | Fraction A | Fraction B | Fraction C | Fraction D |
|---|---------------|-------------|--------------|--------------|--------------|
| Newly laid eggs | 5 | 3.4 ± 0.109 | 15.2 ± 0.208 | 65.1 ± 0.175 | 16.3 ± 0.183 |
| 1 day | 3 | 3.4 ± 0.100 | 15.8 ± 0.329 | 60.9 ± 0.674 | 19.9 ± 0.177 |
| 2 day | 4 | 3.5 ± 0.244 | 16.9 ± 0.274 | 56.2 ± 0.497 | 23.4 ± 0.270 |
| 3 day | 5 | 3.5 ± 0.479 | 16.9 ± 0.625 | 49.2 ± 0.494 | 30.4 ± 0.455 |
| 4 day | 3 | 3.2 ± 0.279 | 17.0 ± 0.229 | 48.1 ± 0.218 | 31.7 ± 0.278 |
| 5 day | 4 | 3.4 ± 0.189 | 16.5 ± 0.075 | 44.0 ± 0.047 | 36.1 ± 0.084 |
| 6 day | 3 | 3.2 ± 0.178 | 16.2 ± 0.328 | 43.0 ± 0.336 | 37.6 ± 0.123 |
| 7 day | 3 | 3.4 ± 0.250 | 16.2 ± 0.556 | 40.5 ± 0.379 | 39.9 ± 0.500 |
| Newly hatched larvae + chorions (Day 8) | 3 | 3.5 ± 0.358 | 16.9 ± 0.201 | 39.6 ± 1.151 | 40.0 ± 1.404 |

day. Fraction C (water soluble nitrogen precipitated by TCA) decreased from 7.4 to 5.7% with a significant decrease on the fourth day. Fraction D (insoluble nitrogen) showed significant changes during the first three days of development, increasing from 77.0 to 80.0%. This fraction was relatively constant from the fourth to the seventh day, and on the eighth day, it increased significantly from 81.0 to 81.8%. This is the only fraction to show a net increase over the entire embryonic period. The 4.8% increase is primarily due to shifts of material from fractions A and C. The major changes occurred during the first three days of development.

The nitrogen content, expressed as per cent total nitrogen, for the fractions determined by the procedure of DelVecchio are given in Table 2. Fraction A (lipid nitrogen), which makes up approximately 3.4%, and Fraction B (water soluble nitrogen not precipitated by TCA), which makes up approximately 16% of the total, show no significant changes during development. Fraction C (water soluble nitrogen precipitated by TCA) decreased from 65.1 to 39.6% with significant decreases on all but the fourth and eighth days. Fraction D (insoluble nitrogen) increased from 16.3 to 40.0% with significant increases on all but the fourth and eighth days. The increases in Fraction D are due entirely to shifts of material from Fraction C.

The results obtained with the seven fraction technique, expressed as per cent total nitrogen, are given in Table 3. Fraction A (water soluble nitrogen precipitated by boiling) decreased during the entire period of development. Significant decreases were found on the first, third, fourth, sixth and seventh days. Fraction B (water soluble nitrogen not precipitated by boiling or by TCA) increased slightly, but significantly during the first two days, and was relatively constant for the remainder of the developmental period. Fraction C (water soluble nitrogen precipitated by TCA) showed significant decreases on the second, third,

TABLE 3. Distribution of nitrogen during the embryogenesis of the mealworm (seven fraction technique). Figures, expressed as per cent total nitrogen, are given with standard errors.

| Age | No. of trials | Fraction A | Fraction B | Fraction C | Total water soluble nitrogen |
|---|---------------|--------------|--------------|--------------|------------------------------|
| Newly laid eggs | 8 | 12.7 ± 0.167 | 14.6 ± 0.209 | 52.7 ± 0.472 | 80.0 |
| 1 day | 8 | 8.6 ± 0.379 | 15.5 ± 0.121 | 52.8 ± 0.718 | 76.9 |
| 2 day | 8 | 7.9 ± 0.056 | 16.0 ± 0.151 | 47.7 ± 0.321 | 71.6 |
| 3 day | 8 | 6.0 ± 0.140 | 16.0 ± 0.176 | 41.9 ± 0.147 | 63.9 |
| 4 day | 8 | 5.1 ± 0.202 | 15.6 ± 0.174 | 41.5 ± 0.234 | 62.2 |
| 5 day | 8 | 4.6 ± 0.310 | 15.6 ± 0.286 | 40.4 ± 0.544 | 60.6 |
| 6 day | 8 | 3.1 ± 0.147 | 15.7 ± 0.131 | 39.9 ± 0.382 | 58.7 |
| 7 day | 8 | 2.2 ± 0.096 | 15.7 ± 0.236 | 38.5 ± 0.229 | 56.4 |
| Newly hatched larvae + chorions (Day 8) | 8 | 2.1 ± 0.088 | 15.9 ± 0.063 | 37.8 ± 0.156 | 55.8 |

| Age | Fraction D | Fraction E | Fraction F | Fraction G | Total water insoluble nitrogen |
|---|--------------|--------------|-------------|-------------|--------------------------------|
| Newly laid eggs | 7.5 ± 0.118 | 7.7 ± 0.258 | 1.9 ± 0.113 | 2.9 ± 0.148 | 20.0 |
| 1 day | 8.1 ± 0.220 | 9.9 ± 0.172 | 1.8 ± 0.101 | 3.3 ± 0.147 | 23.1 |
| 2 day | 9.0 ± 0.110 | 13.1 ± 0.248 | 1.8 ± 0.058 | 4.5 ± 0.157 | 28.4 |
| 3 day | 10.3 ± 0.189 | 16.5 ± 0.099 | 2.2 ± 0.070 | 7.1 ± 0.111 | 36.1 |
| 4 day | 10.0 ± 0.110 | 17.5 ± 0.246 | 1.9 ± 0.048 | 8.4 ± 0.056 | 37.8 |
| 5 day | 11.0 ± 0.383 | 18.1 ± 0.240 | 1.8 ± 0.181 | 8.5 ± 0.216 | 39.4 |
| 6 day | 12.7 ± 0.318 | 18.3 ± 0.457 | 1.8 ± 0.112 | 8.5 ± 0.481 | 41.3 |
| 7 day | 13.6 ± 0.190 | 18.8 ± 0.190 | 1.7 ± 0.031 | 9.5 ± 0.101 | 43.6 |
| Newly hatched larvae + chorions (Day 8) | 14.1 ± 1.240 | 18.4 ± 0.118 | 1.9 ± 0.045 | 9.8 ± 0.120 | 44.2 |

seventh and eighth days. The largest decrease occurred during the second and third days. The sum of the water soluble fractions (A, B and C) decreased from 80.0 for newly laid eggs to 55.8% on day 8. During the same period, Fraction D (water insoluble nitrogen extracted with 10% NaCl) increased from 7.5 to 14.1% with significant increases on all but the fourth day. Fraction E (water insoluble nitrogen extracted with 0.1N NaOH and 0.1N HCl) increased significantly during each of the first four days and remained relatively constant during the last four days. The major increase in this fraction occurred during the first three days when it increased from 7.7 to 16.5%. Fraction F (water insoluble nitrogen extracted with lipid solvents) remained constant at a level of approximately 1.9% throughout the embryonic period. Fraction G (insoluble nitrogen) increased from 2.9 to 9.8% with significant increases on all but the fifth day, while the total water insoluble nitrogen (sum of D, E, F and G) increased from 20.0 to 44.2%. The major shifts are from fractions A and C into fractions D, E, and G. They occur primarily during the first three days.

DISCUSSION

The consistency of the total nitrogen values reported in this paper is in agreement with work done on other insects (Farkas 1903, on *B. mori*; Trowbridge and Bodine 1940, on *M. differentialis*; Rothstein 1952, on *P. japonica*).

A comparison of the results obtained in the present work on the egg of *T. molitor* using the technique of Ludwig and Rothstein with the results of these authors on the egg of *P. japonica* indicates essential differences in nitrogenous composition. Ludwig and Rothstein observed large shifts in nitrogen during development. Water soluble nitrogen precipitated by protein precipitating agents (Fraction C) was found to decrease from 81.4% of the total in newly laid eggs to 10.0% just before hatching. The insoluble nitrogen (Fraction D) increased from 12.9 to 71.8% during the same period. Fraction C may contain certain mucoids and intermediate products of protein hydrolysis, the latter are best described as relatively low molecular weight polypeptides. In the present study, only slight changes are shown in these fractions indicating, that in the mealworm, the nitrogenous substances in the egg are of a more complex nature. With the method of Ludwig and Rothstein this material is denatured during the fractionation procedure, thereby obscuring changes in protein composition that might otherwise be noted.

Employing the technique of DelVecchio (1955), water soluble proteins of a more complex nature, such as albumins, are included in the water soluble extract, and since these substances are precipitated by TCA, they are included in fraction C. The differences in the sizes of fraction C obtained by the two procedures clearly indicates that a large portion of the stored nitrogen in the egg of the mealworm is in the form of relatively complex water soluble protein. Pigorini (1925), in his work on the silkworm, *B. mori*, found that albumins comprised approximately 40% of the nitrogenous reserve of the egg. Needham's (1931) review of embryonic nutrition indicates that albumins serve as one of the primary nitrogenous reserves throughout the animal kingdom. It appears that the apparent absence of large quantities of albumin, and other heat or alcohol-ether denatured proteins, in the egg of the Japanese beetle represents a rather atypical case.

The seven fraction technique introduced in this paper gives a more comprehensive view of nitrogenous composition and metabolism. Fraction A (water soluble nitrogen coagulation by boiling), consisting entirely of albumins, is included in fraction D by the technique of Ludwig and Rothstein, and in fraction C, by that of DelVecchio. Fraction B (water soluble nitrogen not precipitated by boiling or by TCA), corresponding to fraction B in both the technique of Ludwig and Rothstein and that of DelVecchio, may contain amino, and other non-protein nitrogenous compounds such as urea and ammonium salts. Fraction C (water soluble nitrogen precipitated by TCA) may contain mucoids and

intermediate products of protein catabolism. This fraction, along with fraction A, is equivalent to fraction C by the technique of DelVecchio. The intermediate products of protein catabolism are probably equivalent to fraction C in the technique of Ludwig and Rothstein, while more complex materials are probably denatured, and are therefore included in fraction D by their procedure. Water insoluble nitrogen extracted with 10% NaCl, fraction D by the seven fraction technique, is primarily composed of globulins; however, some lipoproteins may also be included. The globulins are contained in fraction D, by the technique of Ludwig and Rothstein and that of DelVecchio, while the lipoprotein would be included in fraction A by both procedures. Fraction E (water insoluble nitrogen extracted with 0.1N NaOH or 0.1N HCl), which may contain nucleoproteins, and fraction G (insoluble nitrogen), containing scleroproteins, are included in fraction D by the technique of Ludwig and Rothstein and that of DelVecchio. Fraction F (water insoluble nitrogen extracted with lipid solvents) may contain proteolipids. This material, along with the lipoprotein extracted in fraction D by this procedure, is included in fraction A by the techniques of Ludwig and Rothstein and of DelVecchio.

In the present study, it was found that approximately 24% of the total nitrogen of the mealworm egg is converted from water soluble to water insoluble material during embryogenesis. By employing the seven fraction technique it was possible to show that the utilization of egg albumin accounted for almost 50% of this material, with the remainder supplied from fraction B. An increase in globulin content accounts for approximately 25% of the increase in water insoluble protein. The increase in fraction E which accounts for 45% of the change in water insoluble nitrogen, may be due to synthesis of new nucleoproteins; however, the exact composition of this fraction is not known. The synthesis of scleroprotein accounts for 30% of the increase in water insoluble protein. These changes are similar to those reported by Pigorini (1925) for the silkworm, *B. mori*. It appears, therefore, that these two organisms have the same general pattern of protein metabolism during embryogenesis.

Literature Cited

- DELVECCHIO, R. J. 1955. Changes in the distribution of nitrogen during growth and metamorphosis of the housefly, *Musca domestica* (Linnaeus). J. N. Y. Ent. Soc., **63**: 141-52.
- FARKAS, K. 1903. Beiträge zur Energetik der Ontogenese. III. Ueber den Energieumsatz des Seidenspinners während der Entwicklung im Ei und während der Metamorphose. Arch. f.d. ges. Physiol., **98**: 490-546.
- HOROWITZ, N. H. 1939. The partition of nitrogen in the developing eggs of *Urechis caupo*. J. Cell. Comp. Physiol., **14**: 189-95.
- LUDWIG, D., AND F. ROTHSTEIN. 1952. Changes in the distribution of nitrogen during the embryonic development of the Japanese beetle (*Popillia japonica* Newman). Physiol. Zool., **25**: 263-68.

- NEEDHAM, J. 1931. Chemical embryology. London: Cambridge University Press.
- . 1942. Biochemistry and morphogenesis. London: Cambridge University Press.
- NIEDERL, J. B., AND V. NIEDERL. 1938. Organic quantitative microanalysis. New York: John Wiley and Sons.
- PIGORINI, L. 1925. Contributo alla conoscenza dei fenomeni chimica dell' uova degli insetti (*B. mori*). Le sostanza proteiche. Ann. d. R. Staz. Bacol. Spec. di Padova, **44**: 1-21.
- ROTHSTEIN, F. 1952. Biochemical changes during the embryonic development of the Japanese beetle (*Popillia japonica* Newman). Physiol. Zool., **25**: 171-78.
- TROWBRIDGE, C., AND J. H. BODINE. 1940. Nitrogen content and distribution in eggs of *Melanoplus differentialis* during embryonic development. Biol. Bull., **79**: 452-58.
- WAGNER, E. C. 1940. Titration of ammonia in presence of boric acid in the macro-, semi-macro-, and micro-Kjeldahl procedures, using methyl red indicator and the color matching end point. Ind. Eng. Chem., Anal. Ed., **12**: 771-72.

RECEIVED FOR PUBLICATION FEBRUARY 16, 1967